

Preliminary Tests of the Toxin Extracted from California Sea Hares of the Genus *Aplysia*¹

LINDSAY R. WINKLER²

DURING A STUDY of the biology of California sea hares (Winkler, 1957), the large amounts of absorbed but unused substances from the sea hare's diet which are to be found in the digestive gland were noted. On the assumption that if there are toxic sea weeds in the diet of the sea hare these toxins might possibly be present in the digestive gland, samples of the exudate from frozen digestive glands on hand were removed with a small pipette and were placed in test tubes. The test tubes were then placed in a boiling water bath for 10 min. After being centrifuged, milliliter aliquots were injected intraperitoneally into 20-gm. C57 black mice. The digestive glands of *Helix aspersa* O. F. Muller were similarly treated as controls. The animals injected with the experimental extract showed almost immediate respiratory symptoms followed by an excitement stage and death in 4 to 6 min. Since neither the controls nor ashed samples reconstituted and injected produced these effects, further study seemed desirable. Certain general aspects of the resulting study are reported here.

It may or may not be significant that Latin and Medieval writers, beginning with Pliny (*ca.* A.D. 60) considered the sea hare to be very poisonous and claimed its use in poisonings during the days of Imperial Rome. An excellent summary of the beliefs and superstitions pertaining to the sea hare is given by Johnston (1850).

MATERIALS AND METHODS

Collections in March, April, and May were made at Doheney Beach near Dana Point, California. Later collections were made from May

through August at Lunada Bay, Palos Verdes, Los Angeles County, California. The former were small specimens between 4 and 6 in., the latter were large breeding specimens measuring to 1 ft. in length. Animals were collected at low tides, packed in wet *Pelvetia fastigiata*, and transported back to the laboratory where they were either immediately dissected or were refrigerated until the next day. In either case, the animals were alive when dissected and no difference in the toxic effect was noted between those dissected immediately and those stored overnight.

The digestive glands were dissected out by making a longitudinal, midpedal cut with scissors in the ventral surface of the foot from the tail to the lip area. The animal was then turned inside out. The digestive gland was removed, including the part of the intestine embedded in the gland and the ovotestis, which is an integral part of the digestive gland complex. The percentage weight of the digestive gland complex to the weight of the intact animals was determined in a certain number of cases. Samples of the crop and/or intestinal contents were preserved in alcohol and studied where it seemed desirable in a rough attempt to ascertain if the diet was responsible for the toxicity. Specimens of *Aplysia vaccaria* Winkler were also collected and dissected for extraction. The digestive glands were stored in glass containers in a deep-freeze and were not thawed until ready for use.

It early became apparent that a more refined method of extraction than the water methods used initially was necessary because of the large amounts of pigments, salts, and apolar materials present in the gland. After much trial and error the following method proved the most satisfactory for large-scale crude extract preparation. Thawed digestive gland (130–150 gm.) is placed in a Waring Blendor. When the gland is thoroughly liquified, 400 ml. of acetone is

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² Department of Pharmacology, College of Medical Evangelists, Loma Linda, California.

slowly added, and after a few minutes the entire mixture is quickly filtered. The residue is then rinsed with an additional 100 ml. of acetone. The acetone extract, containing the tissue "water" from the digestive gland is then placed in the flask of a rotary evaporator, partial vacuum being supplied by an aspirator. As the percentage of acetone is reduced by evaporation, the apolar fraction is thrown out of solution and deposited on the flask wall. When the remaining "water" has been evaporated to about 50 cc., it is placed in a refrigerator overnight, after which it is refiltered and becomes what is referred to as the crude extract.

The routine bio-assay procedure for indicating relative strength of extracts in the toxic principle consists of injecting 1 ml. of an extract intraperitoneally into mice of similar weight and observing the death time to the last heartbeat audible with a stethoscope. The death time serves as a rough indication of the relative toxicity.

Initial LD₅₀ values were obtained by conventional methods using three groups of mice to establish three points on a graph plotting percentage mortality against dose given. The LD₅₀ values were then read from the graph. Subsequent LD₅₀ approximations and those for the chick were determined using a minimum number of animals by the "up and down" method proposed by Dixon (1959). Since the LD₁₋₉₉ range is very narrow, this latter method gave adequate results for the present purposes.

EXPERIMENTAL RESULTS

The average percentage weight of the digestive gland/ovotestis complex of *A. californica* based on 15 specimens was 10.8 per cent, and the range was from 8.4 to 14.0 per cent. Varying amounts of sand were found in the intestines of this species, which influenced the accuracy of the weights. The accuracy was higher for *A. vaccaria*, however, since no sand was found in the intestines. The average percentage based on 5 specimens of the latter species was 19.4 per cent, ranging from 18.0 to 20.3 per cent.

The diet varied between the two collecting sites. At Doheny Beach the diet followed the predominant flora consisting of several coralline

algae and *Hypnea californica*. A considerably wider variety of seaweeds was noted in the Lunada Bay collections. The Lunada Bay sea hares appeared to be more toxic but this may have been the result of more maturity or merely a reflection of variation in the extraction efficiency. No correlation is possible at the present state of the research.

Aplysia vaccaria, a sea hare rather distantly related within the genus, possesses a similar toxin in its digestive gland.

The LD₅₀ was determined for 23-gm. mice and is expressed in grams of digestive gland tissue. Two different batches of raw material were used to obtain two somewhat removed values. Each batch represents the material obtained from 5 to 12 sea hares, depending on the animals' size. One batch collected May 4, 1959, had an LD₅₀ value of 0.65 gm. tissue for a 23-gm. mouse (0.028 gm./gm body weight). Another collected at the same location July 28, 1959, had a value of 0.8 gm. per 23-gm. mouse (0.036 gm./gm weight). However, the difference in the two values may represent only differences in extraction efficiency rather than true variation in toxin concentration.

The LD₅₀ for 3-day-old baby chicks was found to be only slightly less than 25 per cent more than that for mice.

When mice are injected intraperitoneally with the crude toxin of somewhat more than the LD₅₀ dosage, there is an almost instantaneous hyperventilation. Ears are drooped and the mouse usually sticks his nose in a corner of the cage and salivates profusely. After a varying time in which hyperventilation is evident, the mouse starts scurrying about the cage, usually leaving a trail of urine. Perhaps it then returns to its corner or begins to demonstrate occasional muscular twitching which may turn into uncontrolled attempts at movement suggesting a convulsion. This uncontrolled movement may develop in waves and once begun is always terminal. Ataxia and inability to right itself usually develop before or during these uncontrolled movements. The animal passes into a completely relaxed state. The heart continues for some time at a reduced pace, gradually becoming weaker until it can no longer be heard with a stethoscope. The toxin also killed mice

when given by stomach tube at approximately 12 times the intraperitoneal LD_{50} dose.

Helix aspersa O. F. Muller withstood doses of the toxin sufficient to kill several mice with only temporary effects. However, the purple shore crab quickly went into a relaxed state when small quantities were injected at the podial interstices. Since they were presumed dead, they were not observed further.

When frogs are injected with the LD_{50} dose/gm weight for mice, the dorsal appendages become weakened and paralyzed in approximately 5 min. This is followed quickly by relaxation (Fig. 1), complete except for the anterior limbs, which become spastic. The rectus abdominis is also tensed. The muscles controlling the eyes are the last to become paralyzed. A complete deathlike stupor follows, lasting about 15 hr. The first reaction to reappear is the movement of the nictitating membrane and retraction of the eyes. Soon thereafter, when teased in the eye region, an isolated leg twitch may occur, usually in the thigh region. As time progresses,

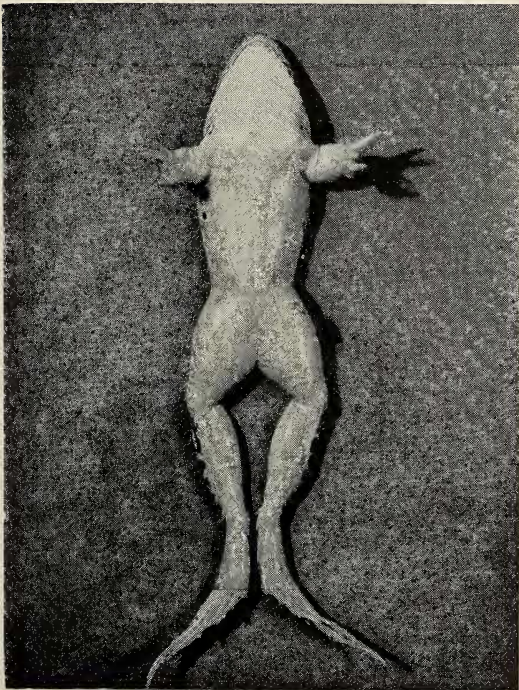


FIG. 1. The reaction of the frog to the toxin of *A. californica*. Note the tensed abdominal muscles and forelegs.

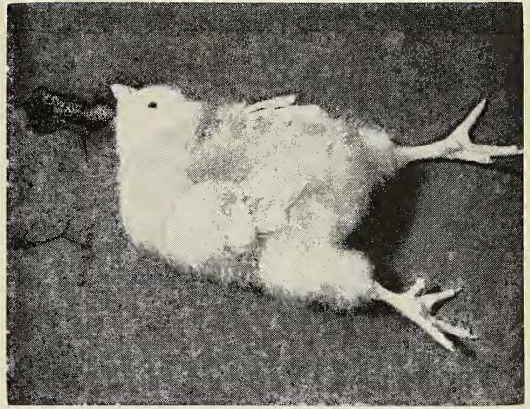


FIG. 2. The reaction of the chick to the toxin of *A. californica*. The toxin was injected subcutaneously over the breast.

teasing produces an initial kick followed by complete immotility for a time. In 3 to 5 hr. more the frogs recover completely.

Three-day-old baby chicks were injected with lethal doses to observe symptoms. Immediate hyperventilation occurred, followed in order by ataxia, relaxation of the wings, and a stretching of the legs (Fig. 2), which were relaxed only terminally. Hypersalivation and difficulty in swallowing were apparent. Respiratory arrest preceded a final convulsive effort before complete cessation of movement.

Rats and guinea pigs show symptoms quite similar to those of the mouse. Kittens, on the other hand, passed through a short but violent writhing and vomiting stage. Hyperventilation, relaxation of the vocal cords and nictitating membrane, and dilation of the pupils followed. Relaxation of forepaws and neck muscles preceded respiratory distress, violent tail wagging, terminal respiratory arrest, and relaxation of the bladder sphincter muscles.

DISCUSSION AND CONCLUSIONS

The common denominator of the lethal symptoms observed seems to be respiratory paralysis with no other noticeable lethal effects. Frogs, though able to survive doses paralyzing their lung respiration for 15 hr. or more, succumbed sporadically to a wide range of much larger doses. This may indicate other less dominant

lethal effects masked by the respiratory arrest. However, respiratory arrest seems to be the limiting factor in birds and mammals, though a suggestion of other contributing effects is noted in the chick. Studies on isolated preparations are now in progress and will be reported later.

At the present stage of the study it is impossible to postulate the function, if any, or the ultimate source of the toxin. The absence of any method for the animal to inject the toxin along with the high dosages required to be effective orally would seem to preclude any defensive use. However, both concentration of the toxin from the seaweed diet or an endocrine function may be considered as possibilities.

SUMMARY

1. The digestive gland of *Aplysia californica* and *A. vaccaria* contain a water- and acetone-soluble toxin.

2. Crude extracts produced muscular weakness and death by respiratory arrest when injected intraperitoneally into various laboratory animals or given orally at about 12 times the IP dose.

3. Frogs survived a respiratory arrest and complete paralysis for 15 hr. When extracts are given in much larger doses, death ensues from causes not yet determined.

4. From observation of these symptoms, it is suggested that the primary lethal effect in mammals and birds is respiratory arrest, though other less dominant lethal effects seem to operate in the frog.

REFERENCES

- DIXON, W. J. 1959. [A later, as yet unpublished, simplification of his method as published by A. W. Kimball, W. T. Burnett, Jr., and David G. Doherty, 1957.] Chemical protection against ionizing radiation. *Rad. Res.* 7: 1-12.
- JOHNSTON, GEORGE. 1850. *Introduction to Conchology; or Elements of the Natural History of Molluscous Animals.* J. Van Voorst, London.
- PLINY, SECUNDUS. ca. A.D. 60. *Historia Naturalis*, Lib. 9, 32.
- WINKLER, LINDSAY R. 1956. *The biology of California sea hares of the genus Aplysia.* Dissertation, Univ. So. Calif., Los Angeles.